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In recent years, it has become increasingly evident that when limbic excitatory transmitter systems are activated by any of a number of generator mechanisms, it results in continuous seizure activity in limbic circuits (limbic status epilepticus) which tends to persist for hours and typically eventuates in disseminated brain damage affecting brain regions hosting the intense seizure discharge activity. Accumulating evidence suggests that excitatory amino acid transmitters (glutamate or related compounds), which are released in excessive amounts during status epilepticus, and are known to have neurotoxic (excitotoxic) properties, may be responsible for the brain damage associated with seizures. Cholinergic mechanisms may also be involved since it is possible to induce persistent seizures and related brain damage by either systemic or intra-amygdaloid administration of cholinergic agonists or cholinesterase inhibitors. Moreover, recent studies have documented that the neurotoxic syndrome produced by cholinergic neurotoxins used in chemical warfare (organophosphorus anticholinesterases) includes persistent seizures and disseminated brain damage. Experiments reported here were undertaken to assess the relative contribution of cholinergic and glutamergic mechanisms in cholinotoxic syndromes and to explore new approaches for protecting the central nervous system against cholinergic neurotoxins.

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INTRODUCTION

This report covers a 4 year period from Oct 1, 1985 to Sep 30 1989. At the beginning of the contract period it was known that systemic administration of kainic acid (KA), an excitatory amino acid (EAA), induces a distinctive neurotoxic syndrome in adult rats consisting first of stereotypic behaviors, then repetitive seizure activity which persists for hours and inevitably is accompanied by brain damage disseminated throughout the limbic system in a pattern coinciding with the termination of fiber pathways involved in the seizure circuitry. It had also been shown that the cytopathology associated with KA-induced seizure activity appeared identical to the distinctive excitotoxic type of cytopathology induced by exposure of neurons to glutamate (Glu) or other EAA *in vivo* or *in vitro* (1,2,3), and that when specific pathways that use Glu as transmitter (for example, the corticothalamic tract or perforant path) are persistently hyperstimulated, either pharmacologically or electrically, it results in acute Glu-like excitotoxic cytopathological changes in dendrosomal neural elements post synaptic to the stimulated pathway (4,5).

A seizure-related brain damage (SRBD) syndrome similar to that induced by KA had also been described in rats following intra-amygdaloid injection of cholinergic agonists such as pilocarpine (pilo) and carbachol or acetylcholinesterase inhibitors, such as physostigmine and neostigmine (6), or following systemic injection of pilo, either alone or preceded by lithium (li) (7). Activation of muscarinic cholinergic receptors had been implicated in the initiation of seizure activity in these cholinotoxic syndromes in that atropine pretreatment prevented both the seizures and related brain damage (8,9). Administration of atropine after the cholinotoxic syndrome had commenced, however, was relatively ineffective in conferring such protection.

Since cholinergic agents caused Glu-like seizure-related cytopathological changes which were indistinguishable from those induced by other convulsant agents, such as KA, or by excessive stimulation of Glu fiber tracts, it was proposed that all such SRBD whether stimulated by cholinergic or non-cholinergic agents, can be viewed as a form of excitotoxin-mediated brain damage in which the offending excitotoxin is endogenous Glu or a related endogenous excitotoxin released by the persistent seizure activity. According to this hypothesis, as applied to cholinotoxic syndromes, the cholinergic agent initiates the seizure activity but is not directly responsible for the toxic destruction of neurons within the seizure pathways. In further support of this interpretation was evidence (6) that when cholinergic agonists were injected directly into the brain in doses sufficient to induce seizures and brain damage disseminated over various brain regions distant from the injection site, this did not result in brain damage at the local injection site. Also in support of this interpretation is evidence generated by the contracting laboratory showing that systemic administration of MK-801, an antagonist of the N-methyl-D-aspartate (NMDA) subtype of Glu receptor, protects neurons against seizure-mediated toxic degeneration associated either with KA treatment or with hyperstimulation of the corticothalamic Glu fiber pathway (10,11). This suggests that in these SRBD syndromes, the cytopathological events may be mediated primarily by the NMDA subtype of Glu receptor.

At the beginning of this contract period, investigators were just beginning to recognize that the devastating neurotoxic syndrome associated with exposure to the organophosphorus cholinesterase inhibitor, soman, often includes persistent seizure activity and may result in brain damage if the animals survived long enough for pathomorphological changes to be expressed; this raised the question whether the soman cholinotoxic syndrome might be viewed either partially or entirely as a seizure-mediated syndrome in which the seizure activity is cholinergically generated but the brain damage results from excessive release of Glu or a related endogenous excitotoxin.

The goals of the proposed research pertained entirely to studies in adult rats and were essentially three-fold: 1. To conduct a systematic evaluation of the SRBD syndrome induced by systemic pilo or li/pilo, using a combination of behavioral, neuropathological, electrophysiological, metabolic and neurochemical methods; 2. To conduct a similar evaluation of the SRBD syndrome induced by systemic soman administration; 3. To evaluate certain drugs for their ability to prevent the soman neurotoxic syndrome. In this report, the work performed during the contract period will be

described and discussed in terms of projects 1, 2 and 3 which correspond to the above 3 goals respectively.

PROJECT #1: EVALUATION OF THE CHOLINOTOXIC SYNDROME INDUCED BY PILO OR LI/PILO

Introduction

In this section we will describe electrophysiologic, metabolic, receptor autoradiographic, neurochemical and neuropathologic studies of pilo and li/pilo-induced SRBD. This series of studies provided an in depth analysis of the onset, propagation and pathological consequences of cholinergically-induced seizures and served as a basis for subsequent analyses of soman-induced cytopathology.

Materials and Methods

General protocol

Adult Sprague-Dawley rats weighing about 300 g, bred and raised in the animal quarters of the Department of Psychiatry at Washington University Medical School on a 12-hour, light-dark cycle with free access to food and water, were used in all experiments. Lithium chloride (li) and pilo (Sigma Chemical, St. Louis, MO) were administered subcutaneously (sc) in one of the following treatment regimens: 1) 3 meq/kg li followed after 24 hr by 30 mg/kg pilo, 2) 400 mg/kg pilo without li pretreatment. In addition, 6 rats were injected sc with 12 mg/kg KA (Sigma Chemical, St. Louis, MO) in order to compare the electrographic and neuropathologic aspects of the KA syndrome with those produced by pilo. Control rats comprised 3 groups: 1) saline only, 2) 3 meq/kg li followed in 24 hrs by saline, 3) saline followed in 24 hrs by 30 mg/kg pilo. All animals that seized persistently for at least 2 hrs and were to survive at least 24 hrs were administered diazepam (10 mg/kg sc) 3 hrs and again 4 hrs after the onset of seizures to arrest the seizure activity, thereby assuring an acceptable survival rate (Price, M.T. and Olney, J.W., unpublished observation). These animals were hand fed with liquid formula until they were able to function without aid.

Electroencephalography

For electrographic studies, animals were anesthetized with halothane and concentric bipolar electrodes (Rhodes Medical Instruments, Tujunga, CA), or bipolar stainless steel twist electrodes were stereotactically placed in various regions according to the coordinates of Pellegrino et al. (12). Depth sites monitored in various animals included the hippocampus, entorhinal cortex, amygdala, lateral septal nucleus, caudate-putamen, nucleus accumbens, ventral globus pallidus, substantia nigra and ventrobasal thalamus. Surface electroencephalographic (EEG) activity was recorded from teflon-coated stainless steel wires implanted in the inner table of bone above the motor cortex. Additional stainless steel wires were positioned adjacent to the frontal sinus and served as ground and indifferent references. All electrodes were led to Amphenol pins in a connector board and the entire apparatus was affixed to the skull with jeweler's screws and dental acrylate. Each animal had 2-4 sites monitored. After surgery, animals were housed in individual plexiglass cages and were allowed 1 week's recovery before experimental manipulation. Electrode placements were confirmed histologically at the termination of the experiment.

At the time of study, animals were connected to a Grass Model 7D polygraph and EEG signals were amplified and displayed using 7 P5 AC preamplifiers and driver amplifiers. Recordings were obtained from 30 min prior to 6 hrs after drug injections.

2-Deoxyglucose Autoradiography

For metabolic experiments, the quantitative [^{14}C]-2-deoxyglucose autoradiographic technique of Sokoloff et al. (13) was used to measure local cerebral glucose utilization. Prior to experimentation, animals were fasted overnight. On the day of an experiment animals were anesthetized with halothane and polyethylene catheters were inserted into the femoral artery and vein. The animals were then loosely restrained for purposes of restricting movement. Body temperature was monitored via a rectal probe and kept at 36-37° C with a heat lamp. Animals were allowed to recover from anesthesia for 4 hrs before experimentation.

At the time of experiment, animals were injected with pilo sc. After the first episode of forelimb clonus, [^{14}C]-2-deoxyglucose (2-DG) (60 mCi/kg) was administered by intravenous infusion over 30 sec. Timed 0.1-ml arterial blood samples were taken at 1/3, 3/4, 1-1/4, 2, 3, 5, 7-1/2, 12, 20, 30 and 40 min after 2-DG administration. The blood samples were centrifuged immediately and plasma radioactivity and glucose concentrations were measured. Animals were anesthetized with pentobarbitol 45 min after 2-DG administration and perfused with phosphate-buffered 1% paraformaldehyde fixative via an intracardiac cannula. The brains were rapidly removed, frozen and sectioned in a cryostat.

Brain sections (20 μm) were mounted with [^{14}C]-methyl-methacrylate standards (Amersham, Arlington Heights, IL) and exposed to Kodak X-AR film for 7 days. Brain radioactivity and local glucose utilization were determined in brain regions of interest by quantitative autoradiography using a computerized densitometer. Separate measurements of left- and right-sided structures were made and the means of these measurements were pooled for subsequent analysis. Comparisons of experimental animals and controls in all experiments in this report were made using serial t-tests. Differences at a 95% or greater confidence level were considered significant.

Histopathology

Animals were killed by intracardiac perfusion-fixation under halothane anesthesia with phosphate buffered 1% paraformaldehyde and 1.5% glutaraldehyde 4 hrs after the initiation of seizure activity. The brains were removed and cut into 1-mm slabs which were additionally fixed by immersion in 1% osmium tetroxide for 24 hrs. These slabs were processed through graded concentrations of ethanol, cleared in toluene and embedded in Araldite. For light microscopy, 1-μm sections were cut on an MT-2 Sorvall Ultratome and stained with methylene blue-azure II. Regions of interest for ultrastructural analysis were sectioned from the same blocks. Silver-gold sections were mounted on a formvar film suspended over a 1 x 2 mm slot grid, stained with lead citrate and uranyl acetate and viewed in a JEOL 100B transmission electron microscope.

Receptor Autoradiography

Seventy five li/pilo treated adult female Sprague Dawley albino rats were included in this phase of the study. After various survival times (4 or 24 hrs; 1,2,3,4 or 8 wks), animals were sacrificed by decapitation; their brains were quickly removed and frozen in powdered dry ice, then coronally cut into 10 uM sections on a Bright cryostat. In some instances, brains were maintained at -80° C before sectioning.

Glu receptor binding was performed as previously published (14). Sections were mounted on acid-washed gel-coated coverslips and preincubated in 50 mM Tris acetate buffer pH 7.25 at 4° C for 60 mins to remove endogenous Glu, air-dried and placed in xylene for 10 mins. The sections were again air-dried, stored at -20° C overnight (optional), then incubated for 10 mins at 4° C in Tris acetate buffer containing 25-1000 nM [^{3}H]-Glu. Nonspecific binding was studied by adding 0.5-1 mM unlabeled Glu to the incubation medium. Sections were rinsed for 3 secs in each of a graded series of ethanol/buffer solutions (30,50,70,95 and 100%), air-dried, exposed against ultrofilm in lighttight cassettes and stored at 4° C for 7 days. The films were then developed and the density of Glu binding in various brain regions measured with the Bioquant microdensitometric image analysis

system (R&M Biometrics, Nashville, TN). The following brain areas were analysed at 5 coronal levels: cingulate, frontal, retrosplenial, piriform, parietal, temporal, occipital and insular cortices; caudate putamen; nucleus accumbens; nucleus of the lateral olfactory tract; CA-1, CA-2 and CA-3 regions of hippocampus; dentate gyrus; substantia nigra; dorsolateral and ventroposterior thalamus; and medial and basal nuclei of amygdala. Kinetic constants were determined by Scatchard/Rosenthal analysis. Significance of differences between control and li/pilo-treated brains was determined by Student's 2-tailed t-test.

Binding to muscarinic cholinergic receptors was determined by a protocol the same as that just described with the following exceptions: preincubation of sections was in xylene only; incubation media consisted of 0.25-2 nM [^{3}H]-QNB (1-quinuclidinyl benzilate) in buffer with or without 20 uM atropine sulfate added to study nonspecific binding and sections were rinsed 3 times for 20 secs in ethanol-free buffer.

In both [^{3}H]-Glu and [^{3}H]-QNB binding assays, nonspecific binding was negligible; i.e. it was below the level of sensitivity of our Bioquant system, even if films were exposed for 4 wks.

Neurochemistry

For all neurochemical experiments, rats were allowed to survive 1-12 weeks following li/pilo-induced seizure activity. At time of sacrifice the animals were decapitated and their brains rapidly removed. Samples of neocortex, hippocampus, striatum, thalamus and piriform cortex-amygdala were dissected out, weighed and homogenized in ice-cold 50-mM Tris HCl buffer to which Triton X-100 (0.2%) was added. The homogenate (150 mg/ml) was centrifuged (10,000 g for 10 min at 4° C). The resulting supernatant was collected and assayed in triplicate for cholineacetyltransferase (CAT) and glutamic acid decarboxylase (GAD) by the methods of Fonnum (15,16). Unless otherwise noted, all chemicals were obtained from Sigma Chemical, St. Louis, MO.

CAT Assay Fifty µg homogenate was incubated in medium containing 20mM ethylenediamine tetraacetic acid, 300 mM sodium chloride, 8mM choline chloride, 0.2% Triton X-100, 100mM physostigmine and 200mM [^{3}H]-acetyl coenzyme A (ICN Irvine, CA) in 50mM sodium phosphate buffer, pH 7.4, in a total volume of 82 µl for 30 min at 37° C. To stop the reaction, incubation tubes were placed inside 20-ml liquid scintillation counting vials and the contents washed into the larger tube with 5 ml 100mM sodium phosphate buffer, pH 7.4 at 25° C. Two ml of a saturated solution (1.5%) of sodium tetraphenyl borate in washed heptanone was then added, and the scintillation vials were tightly capped and vigorously shaken for 30-45 sec to extract the [^{3}H]-ACh salt into the organic phase. Ten ml of toluene-based fluorescent beta scintillation counting cocktail was added and radioactivity determined by a Packard TriCarb liquid scintillation analyzer. Control tubes contain either 1) no homogenate or 2) homogenate but no physostigmine and have 1 unit acetyl cholinesterase added.

GAD Assay Homogenate (150µg) was placed in 1µl medium containing 2 mM L-[1-^{14}C]-Glu, 0.05% bovine serum albumin, 0.2% Triton X-100, 1 mM mercaptoethanol and 0.4 mM pyridoxal phosphate in 50 mM sodium phosphate buffer pH 6.5. Each tube was filled with nitrogen gas and carefully sealed with 50 µl 2N sulfuric acid and then connected by a 6-cm length of tygon tubing to a second tube containing 50 µl hyamine hydroxide. Pairs of tubes were incubated for 60 min at 37° C. The acid was then flicked down into the homogenate mixture and the tubes remained at 25° C for 45 min to allow acid-generated gaseous [^{3}H]-carbon dioxide to be captured by the strong base. The hyamine hydroxide tubes were then placed in 20-ml liquid scintillation counting vials and their contents washed out with 2 ml 95% ethanol. Ten ml of a toluene-based fluorescent liquid scintillation counting cocktail was added and radioactivity determined by a Packard TriCarb liquid scintillation analyzer. Control tubes contained no homogenate or contained homogenate which had been boiled for 10 min.

Results and Discussion

Electrophysiologic, Metabolic and Neuropathologic Studies

Ten different surface and depth sites were monitored in 25 animals. Prior to pilo administration, no spiking or organized electrographic seizures were observed in any of the animals. The onset of seizure activity after pilo administration in both pilo and li/pilo treated rats is documented electrographically in Figure 1 which also depicts onset of seizures in an animal treated with KA for comparison. Analysis of depth electrode recordings revealed that the nucleus accumbens was the site where seizure activity was first detectable in high-dose pilo or li/pilo treated rats. This is in contrast to the KA syndrome in which seizure activity was first detected in the hippocampal leads. Almost immediately after onset of seizure activity in the nucleus accumbens of pilo or li/pilo rats, spiking began to appear in numerous other limbic and related brain regions where it rapidly evolved into organized electrographic seizures. Once initiated, continuous electrographic seizure activity persisted for hours.

Increased glucose utilization was found in most brain regions during the period of continuous seizure activity. The greatest increases were found in the ventral pallidum, globus pallidus, hippocampus, entorhinal cortex, amygdala, lateral septum, substantia nigra, ventrobasal and mediodorsal thalamus and frontal motor cortex (see Table 1). Animals sustaining seizures displayed a disseminated pattern of neural degeneration not involving globus pallidus or ventral pallidum but otherwise coinciding with the above pattern of enhanced glucose utilization. No consistent correlation was observed between the pattern of brain damage and known regions of high muscarinic cholinergic receptor density. Ultrastructurally, there was massive swelling of neuronal dendrites and somata and sparing of axons (Fig. 2). Some neurons underwent dark cell changes (vacuolar condensation) which was frequently accompanied by swelling of perineuronal glial cells. These are the same cytopathologic changes typically associated with various other sustained seizure syndromes and they also are essentially identical to the excitotoxic type of damage Glu is known to cause (3).

The cholinergic SRBD syndrome closely resembled that induced by systemic KA, especially in behavioral aspects, but there were potentially significant differences in electrophysiologic and metabolic manifestations. During KA seizures, electrographic changes were first detected in the hippocampus, whereas they were first detected in the ventral forebrain (nucleus accumbens) region in pilo seizures. Pilo also induced metabolic activation of ventral forebrain sites not activated by KA. The cytopathology associated with the two syndromes appeared identical in type and similar but not identical in pattern, the cholinergic model being characterized by much greater neocortical and slightly less hippocampal damage.

By neuropathologic, electrophysiologic or metabolic criteria, the cholinotoxic syndrome induced by li/pilo (3 meq/kg plus 30 mg/kg) was indistinguishable from that induced by high dose pilo (400 mg/kg) alone. Moreover, the type of cytopathology observed is indistinguishable from that we have described in association with other seizure syndromes, which, in turn, is indistinguishable from the known excitotoxic effects of Glu (3). This is consistent with the interpretation that brain damage associated with pilo or li/pilo seizures may stem from a secondary activation of the glutamergic excitatory transmitter system.

While the neurotoxic syndromes produced by li/pilo and high dose pilo treatment appear to be essentially identical, we are impressed that the former provides substantial advantages over the latter for studying mechanisms of cholinergic neurotoxicity. It consistently results in status epilepticus and a predictable pattern and degree of acute brain damage without a high incidence of acute mortality; this permits one to harvest groups of brain-damaged animals surviving weeks or months after seizure so that biochemical and receptor binding studies can be performed on their brains. After exhaustive efforts, in fact, we concluded that it was not feasible to conduct such studies on high dose pilo animals because all such animals died within hours or a few days of treatment, even if given large doses of diazepam and special nursing care in the post treatment period.

Autoradiographic receptor binding studies

The relative densities of [^{3}H]-Glu and [^{3}H]-QNB binding following li/pilo treatment as compared to age-matched controls is illustrated in Fig. 3. In summary, both [^{3}H[-Glu and [^{3}H]-QNB receptor binding were uniformly reduced during early survival periods (4-24 hrs following li/pilo treatment) in nearly every brain region evaluated, including regions that do not typically sustain brain damage. The reductions in [^{3}H]-Glu and [^{3}H]-QNB binding that were already evident at 4 hrs became maximal in the 4-hr to 1-wk period, then gradually returned toward control values by the 8 wk post-treatment period. This pattern was seen in all brain regions except those that sustained the most severe brain damage; in severely damaged regions, binding of both ligands remained severely depressed in the 1-3 wk interval and recovered only moderately in the 4-8 wk time period. In several brain regions that were not damaged, such as the nucleus accumbens and substantia nigra, a substantial reduction in both [^{3}H]-Glu and [^{3}H]-QNB binding at 4-24 hrs progressively converted to a significant increase in binding of both ligands at 4-8 wks.

Scatchard analysis of the piriform cortex in 6 brains within 3 wks of li/pilo treatment (Table 2) demonstrated a 36% decrease in B_{max} of [^{3}H]-QNB binding with no apparent change in the K_d. Considering the profound brain damage in piriform cortex following li/pilo treatment, a 36% loss in receptor number is certainly not surprising. It is interesting that the sensitivity of the remaining cholinergic receptors did not change. In contrast, the B_{max} for [^{3}H-]Glu binding did not change while the K_d increased 40%. Since the piriform cortex clearly sustained a loss of neurons and therefore a loss of glutamate receptors, the unvarying B_{max} would suggest that new Glu receptors were either generated or unmasked in the piriform cortical region to compensate for those lost, but the increase in the K_d indicates that these new receptors are relatively insensitive to Glu as compared to the previous population, and therefore Glu binding was notably reduced.

Neurochemical Studies

In the hippocampus, piriform cortex-amygdala and thalamus of li/pilo rats, GAD activity was significantly decreased in the 1-3-wk period following treatment but returned to and remained at control levels in the 4-12-week interval (Table 3). In the striatum and neocortex of li/pilo rats, GAD activity was not different from control levels throughout the 12 weeks following treatment. CAT activity was significantly decreased in striatum and piriform cortex-amygdala but was unchanged in hippocampus and thalamus throughout the 12-week period following li/pilo treatment. In the neocortex, CAT activity was elevated in the 1-4-wk-interval and returned to control levels by 12 wks following li/pilo treatment.

These findings do not provide any immediate insights into the mechanism of neurotoxicity, although they are entirely consistent with the excitotoxic hypothesis, since the various neural elements destroyed by the excitotoxic process do not have anything in common except that they receive excitatory amino acid inputs. Thus, one might expect loss of either GAD- or CAT-containing neurons that lie postsynaptic to glutamergic terminals. If such neurons are intrinsic to a region in which excessive seizure-mediated release of Glu occurs, they may be destroyed. If such neurons project to other brain regions, there will be a loss of GAD or CAT both in the region of intrinsic neuronal loss and in the terminal fields. An increase in neocortical CAT concentrations for several weeks following li/pilo treatment is interesting and presumably reflects some kind of reactive change in the cholinergic system induced by li/pilo treatment and/or the associated seizures.

PROJECT #2: EVALUATION OF THE CHOLINOTOXIC SYNDROME INDUCED BY SOMAN

Introduction

In general, the aims of the experiments under project #2 were to treat adult rats with soman and study the behavioral, electrophysiological and neuropathological consequences, looking in

particular for ways in which the soman syndrome resembles or differs from other SRBD syndromes. The methods employed were the same as those described above for evaluation of the pilo or li/pilo cholinotoxic syndromes except that the metabolic, receptor autoradiographic and neurochemical analyses were not performed. The reason for not including these exceedingly time consuming analyses is that we learned from the pilo studies in project #1 that the evidence obtained from these three approaches, especially the metabolic and receptor autoradiographic approaches, tended to be highly complex and to defy meaningful interpretation; therefore, it did not achieve its intended purpose of clarifying the relative roles of the cholinergic and glutamergic transmitter systems in cholinotoxic syndromes. Moreover, none of the three methods provided any new insights not already being obtained from behavioral, electrophysiological and neuropathological approaches.

Materials and methods

Adult Sprague Dawley rats (n = 76), both male (n = 36) and female (n = 40), were treated by one of two regimens: either they were pretreated with 3 meq/kg sc li, then given 125 μg/kg sc soman intraperitoneally (ip) 24 hrs later, or they received 125 μg/kg ip soman without li pretreatment (except in pilot studies in which a range of soman doses was evaluated). All animals were observed for 4 hours for seizure-related behaviors, then sacrificed under deep anesthesia (150 mg/kg pentobarbital ip) by intracardiac perfusion with 1.5% glutaraldehyde plus 1% paraformaldehyde in phosphate buffer. Some rats that appeared to be dying prior to 4 hours were rapidly anesthetized and perfused when they reached a terminal state. After 15 min of percardiac perfusion fixation, brains were removed, sliced and postfixed in 1% osmium tetroxide, dehydrated in a graded series of ethanols, cleared in toluene and embedded in Araldite. Thin sections for light microscopy and ultrathin sections for electron microscopy were cut on Sorvall ultramicrotomes and stained with methylene blue/azure II for light microscopy, or with lead citrate and uranyl acetate for ultrastructural examination.

Results

Among the rats (n = 34) that received only soman, 27% developed status epilepticus; among those (n = 42) that received li plus soman, 41% developed status epilepticus. The incidence of seizures was the same for males and females in the two treatment groups. The behavioral syndrome displayed by these animals, regardless of whether they received only soman or li plus soman, was indistinguishable from that which has been described for animals treated with li and pilo, with the exception that the response to soman was less predictable and tended to have all or none characteristics. Whereas essentially 100% of animals treated with li and pilo display persistent seizures which consistently result in moderately severe disseminated brain damage, over 50% of rats treated with soman or li plus soman displayed no seizure-related behaviors. However, once a soman-treated animal began seizing, it inevitably continued to display persistent seizure activity which increased in intensity and caused some rats to reach a terminal state prior to the end of the designated 4 hour observation period. In addition, an occasional animal died quite suddenly only a few minutes after treatment with soman, whereas sudden deaths are not a characteristic of the li/pilo syndrome.

The failure of some rats to respond to soman can only be explained on the basis of individual biological differences among animals, since in pilot experiments we found that rats that showed resistance to soman at a given dose (125 μg/kg sc) continued to show resistance even if large booster doses were given. However, in the same experiment using the same solution of soman, other rats responded to the initial dose (125 μg/kg sc) with a full display of seizures which become intractable and resulted in devastating brain damage.

The brains of all animals, including those that seized and those that did not, were examined by light microscopy and a perfect correspondence was found between those that displayed status epilepticus and those that sustained brain damage. None of the rats that failed to seize following treatment with soman or li plus soman had neuropathological changes in any brain region. All of those that seized (except for two that died abruptly after the initial seizure episode) had acute cytopathological

changes in the same brain regions typically affected by li/pilo, viz the frontoparietal neocortex, piriform cortex, hippocampus, lateral septum, several amygdaloid and thalamic nuclei and the substantia nigra. The cytopathological reaction in each of these brain regions was examined by electron microscopy and found to be identical to that induced by other agents, either cholinergic or non-cholinergic, that cause persistent seizure activity. In affected brain regions, there was edematous swelling of neuronal somata and dendrites as well as clumping of nuclear chromatin leading to nuclear pyknosis (Fig. 2). Some neurons underwent dark cell vacuolar degeneration and this type of neuronal response was often accompanied by massive glial swelling. All of these cytopathological changes have been described as a feature of SRBD syndromes induced by non-cholinergic agents, or as a feature of the brain damage induced in the hypothalamus following sc administration of Glu or related excitotoxins to mice or monkeys (17,18,19,20).

Discussion

These findings strongly suggest that the brain damage induced by soman is seizure-mediated damage; therefore, a primary strategy for preventing such damage would be to develop methods for arresting soman-induced seizure activity. Although various anti-convulsants such as diazepam might be of some value, more promising agents, in principle, would be antagonists that act at the cholinergic receptors where soman causes acetylcholine to accumulate.

Our findings also are entirely consistent with the assumption that an excitotoxic mechanism may underlie the brain damage induced by soman. Therefore, an additional potentially promising strategy for preventing such damage would be to use anti-excitotoxic agents, i.e., agents that antagonize EAA receptors. Our recent finding (10,11) that NMDA antagonists are effective in protecting against other forms of SRBD, raises the possibility that anti-excitotoxic agents that act at NMDA receptors might protect against soman-induced brain damage.

PROJECT #3: STRATEGIES FOR PROTECTING AGAINST SOMAN NEUROTOXICITY

Introduction

One of the research goals specified in our Army contract was to test certain agents for efficacy in protecting the central nervous system against soman neurotoxicity. Agents specified for testing were atropine, a well known anti-cholinergic drug, diazepam, an anti-convulsant that dampens KA-induced seizures, diacetyl monoxime (DAM), an agent which reactivates acetylcholinesterase and D-2-amino-5-phosphonovalerate (D-APV), a competitive NMDA antagonist. It was understood that the Army would supply us with gram amounts of the latter costly compound. When the Army source of D-APV did not materialize, we agreed to an alternate plan of studying MK-801, a very potent noncompetitive NMDA antagonist, which was generously donated to us by Merck, Sharp and Dohme since the Army contract was drafted (and which penetrates blood-brain barriers much more freely than D-APV). Unless otherwise noted, all drugs discussed in the following sections were obtained from Sigma Chemical (St. Louis, MO).

Atropine, diazepam, DAM

We tested atropine (25-100 mg/kg ip), diazepam (10 mg/kg ip) and DAM (100 mg/kg ip), administered either before, after or both before and after soman, and the results pertaining to any of these agents were not promising enough to warrant further study. In particular, being aware of the work of others in this field (21), we were concerned that even if a treatment regimen using some combination of these agents could be shown to be at least partially effective in protecting against soman neurotoxicity, it would very likely require that at least some portion of the treatment regimen be initiated prior to soman administration. Therefore, instead of duplicating the efforts of others in the field who are currently testing complicated pretreatment regimens, we decided to invest our energies in the search for new agents that might provide significant neuroprotective benefits even when administered after exposure to soman.

MK-801

During the last year of the contract period, we conducted extensive studies aimed at evaluating the ability of MK-801 to modify the neurotoxic syndrome induced by soman or pilo. However, in our initial evaluation, we found that MK-801 appeared to potentiate rather than diminish soman cholinotoxicity. Then we examined the effects of MK-801 on pilo neurotoxicity and found that it markedly potentiates the neurotoxicity of this cholinergic muscarinic agonist. For example, the threshold dose of pilo for inducing a SRBD syndrome is approximately 380 mg/kg sc but if the animal is either pre-treated or post-treated with MK-801 (1 mg/kg sc), it requires only 75 mg/kg sc of pilo to reliably induce a SRBD syndrome. Our current interpretation is that MK-801 potentiates pilo or soman neurotoxicity in rats by lowering the threshold for cholinergically-induced seizures. Braitman has reported (22) that MK-801 (5 mg/kg ip), when administered 30 min prior to soman, blocks the seizures and markedly improves the recovery and survival rate of soman-treated guinea pigs. However, his animals were also pretreated with pyridostigmine and in addition received atropine methylnitrate and pralidoxime chloride 30 sec after soman injection. We have observed that if atropine is administered in combination with MK-801 to soman-treated rats, this partially counteracts the seizure potentiating action of MK-801 and permits MK-801 to exert neuroprotective activity in some brain regions. However, it is a very mixed picture which appears to reflect the net effect of two actions of MK-801, one being anti-convulsant and the other being proconvulsant, plus an action of atropine that modifies (partially) the proconvulsant action of MK-801.

In other studies (10), we have observed that MK-801 protects against much (but not all) of the SRBD induced by KA even though it does not eliminate the electrographically recordable seizure activity in the neuroprotected brain regions. We interpret this as evidence for the involvement of NMDA receptors in the brain damage induced by KA and as evidence that blockade of NMDA receptors can have neuroprotective effects vis a vis non-cholinergically induced SRBD. However, in the syndromes induced by soman or pilo, muscarinic cholinergic receptors play a primary role in initiation of seizure activity, and MK-801 has a potentiating action on cholinergically induced seizures which complicates the neuroprotective action it might exert through NMDA receptors. We are continuing to study the interactions of MK-801 with cholinergic mechanisms; however, in view of the complex interplay of mechanisms witnessed thus far, it seems unlikely that any simple formula can be derived for the incorporation of MK-801 into a treatment or prophylactic regimen for protection against soman neurotoxicity. Moreover, other research we have been conducting (next paragraph) raises additional questions about the suitability of MK-801 as a neuroprotective agent.

Recently we found that MK-801, at rather low doses (0.2-0.4 mg/kg sc), causes acute pathomorphological changes in certain neurons in the posterior cingulate and retrosplenial portions of the rat cerebral cortex. The cytoplasm of the affected neurons develops vacuoles that appear to originate from saccules of endoplasmic reticulum and these vacuoles engulf and appear to degrade mitochondria so that the cytoplasmic compartment becomes devoid of mitochondria. A complete description of this MK-801-induced neurocytotoxicity syndrome was published recently in Science (23). In other experiments, we have observed that cholinergic agonists such as pilo appear to potentiate this neurocytotoxic action of MK-801 in that the reaction occurs at much lower doses of MK-801 if the rat is pretreated with pilo. Although we have not examined whether soman might potentiate the neurotoxic action of MK-801 on cingulate/retrosplenial neurons, this is a very likely possibility that must be taken into consideration in evaluating the suitability of MK-801 as an agent for protecting against soman neurotoxicity. In view of these several undesirable properties of MK-801 and the likelihood that any agent in this class would have PCP-like psychotomimetic side effects, we turned our attention during the past year to another class of agents that seems much more promising.

Other strategies

In 1987, we discovered (24) that certain anti-Parkinsonian agents with known anti-cholinergic properties, such as procyclidine, ethopropazine and trihexyphenidyl, also are effective in preventing NMDA neurotoxicity in the chick embryo retina. Since an agent with combined anti-cholinergic and NMDA antagonist properties, in principle, might be ideally suited for antagonizing both of the

major mechanisms underlying soman neurotoxicity, i.e., the cholinergically mediated seizure activity and NMDA receptor-mediated neurotoxicity, we conducted a series of experiments aimed at evaluating this prospect. Although this series of experiments is not part of the research supported by our Army contract, the results are clearly relevant to the Army's mission and therefore are described herein. Initially, we used procyclidine for these studies since it was the most potent NMDA antagonist identified in our prior study of anti-Parkinsonian agents. In some experiments, we studied the ability of procyclidine to prevent li/pilo neurotoxicity and in others its ability to prevent soman or li/soman neurotoxicity. In some of these experiments, we evaluated the ability of procyclidine to prevent the electrophysiological as well as behavioral and neuropathological consequences of soman treatment. Finally, since procyclidine proved quite effective in protecting against either soman or li/pilo neurotoxicity, we explored the possibility that various other anticholinergic agents (with or without NMDA antagonist properties) might have similar anti-cholinotoxic properties.

Procyclidine protection against li/pilo cholinotoxic syndrome

Adult male Sprague Dawley rats (300-400 g) were treated with li (3 meq/kg sc). One day later they were divided into two groups: one group was treated with procyclidine (75 mg/kg ip) or an equivalent volume of saline 15 min *prior* to pilo (30 mg/kg sc) and the other received the same dose of procyclidine or saline 15 min *after* pilo. Rats were observed over a 4 hour period for behavioral signs of neurotoxicity, including preconvulsive signs such as facial grimacing, head nodding, eye blinking, wet dog shakes or evidence of convulsions, including rearing on hind limbs with clonic movements of the head and forelimbs. After 4 hrs, they were anesthetized and perfused through the left cardiac ventricle and ascending aorta with an aldehyde fixative solution for 15 min, then the brains were removed from the skull and processed for histopathological evaluation by methods described above for light and electron microscopy (17,19).

The results were as follows: In the group that received procyclidine *prior* to pilo, all of the saline controls (rats that received li/pilo but not procyclidine) displayed the full syndrome of preconvulsive and convulsive symptoms with persistent seizure activity being present for the majority of the 4 hour observation period. All of these rats (n = 6) had severe brain damage affecting the cerebral cortex, hippocampus, amygdala, piriform cortex, thalamus, lateral septum and substantia nigra. None of the experimental rats (li/pilo + procyclidine) displayed either preconvulsive or convulsive signs and none (n = 6) sustained brain damage. In the group that received procyclidine 15 min *after* pilo, all of the saline controls (n = 6) exhibited a full behavioral syndrome, including persistent seizures, and all sustained disseminated brain damage. Most of the experimental rats had begun to seize before procyclidine was administered, but all convulsive behavior disappeared within 10 minutes after procyclidine administration and all of these rats (n = 6) escaped brain damage.

In view of evidence that procyclidine has NMDA antagonist properties (24) and the recent finding that other NMDA antagonists such as MK-801 can cause cytopathological changes in neurons in the posterior cingulate and retrosplenial cerebral cortices (23), these brain regions were examined in the experimental rats (n = 12) from the above two experiments, i.e., rats that received procyclidine (75 mg/kg ip), and there was no evidence of the vacuolar type of cytopathology that has been described following MK-801 treatment.

Procyclidine protection against li/soman cholinotoxic syndrome

A major problem in studying the soman cholinotoxic syndrome is the marked individual variation in sensitivity of experimental animals. Administering li 24 hours prior to soman causes a moderate, but consistent, increase in the percentage of animals susceptible to soman neurotoxicity. Therefore, in our initial effort to evaluate the possibility that procyclidine might protect against the neurotoxic effects of soman, we pretreated the rats with li prior to soman. Also, in this study we adopted a new protocol in which the neuroprotective drug (procyclidine) was not administered until the rats began displaying obvious behavioral signs of seizure activity.

Adult male Sprague Dawley rats (350-425 g) were pretreated with li (3 meq/kg sc) and 24 hrs later given soman (125 μg/kg sc), then observed for symptoms; animals that began convulsing were treated immediately either with saline or a single dose of procyclidine (75 mg/kg ip); animals that did not convulse, received no further treatment. All animals were anesthetized and killed 4 hours after soman treatment and their brains examined histologically by methods described above. Rats that did not seize (n = 28) did not have any brain pathology. All rats that seized and received saline (n= 8) had severe disseminated brain damage. Rats that seized and received procyclidine (n = 12), stopped seizing within 5 to 15 minutes; all of these rats escaped brain damage.

Using procyclidine to establish a soman sensitive rat colony

After observing that procyclidine could protect rats against soman neurotoxicity when administered after onset of seizures, we decided to exploit this neuroprotective effect to establish, by an inbreeding program, a colony of soman-sensitive rats. For this purpose, we treated 4 month old male and female Sprague Dawley rats with soman (125 μg/kg ip) and observed the animals for seizures. Those that seized (about 40%), were identified as soman-sensitive and were treated immediately with procyclidine (75 mg/kg ip). This arrested the seizure activity within 10 min and allowed the rats to survive and be used as breeding stock to develop a colony of soman-sensitive rats. This inbreeding effort was reasonably successful in that 88% of the F1 generation responded to 125 μg/kg soman by developing persistent convulsions within 15 min.

Procyclidine neuroprotection against soman cholinotoxic syndrome

In the study described above in which procyclidine was shown to prevent soman-induced brain damage, the rats had been pretreated with li. Using the F1 generation offspring of inbred soman-sensitive rats, we tested the ability of procyclidine to prevent the neurotoxic syndrome caused by soman alone (without li pretreatment). Of 64 F1 generation rats that were treated with soman, then with procyclidine as soon as they began seizing, 2 died before procyclidine could stop seizure activity, 1 had mild damage in the hippocampus and 61 were completely protected from the devastating brain damage ordinarily caused by soman (Fig. 4).

Electroencephalographic evidence that procyclidine arrests soman-induced seizures

Electrographic recordings were obtained from the brains of 3 rats in the above study. Two days prior to the study, depth electrodes were implanted in the neocortex, hippocampus and piriform cortex of these rats. On the day of the study, they were challenged with 125 μg/kg soman and observed until they seized, then given procyclidine (75 mg/kg). Electrographic recordings from one of these rats (characteristic of all three) are shown in Fig. 4. These recordings, obtained from all three leads prior to and for 4 hours following drug treatments, revealed normal electrical activity prior to soman administration (Fig. 4A) and severe seizure activity induced by soman (Fig. 4B) which was totally arrested within 5 min after procyclidine treatment (Fig. 4C). No evidence for return of electrographic seizure activity was seen over the 4 hour recording period in any of these 3 rats (Fig. 5).

Several anti-cholinotoxic agents compared

It is not clear whether the ability of procyclidine to protect against soman neurotoxicity stems soley from its anti-muscarinic properties, its NMDA antagonist properties or from both. To explore this, we screened other agents with anti-muscarinic activity, including some which are also NMDA antagonists, for ability to protect against the cholinotoxic syndrome induced by combined li/pilo treatment. We chose the li/pilo approach as it is a very reliable method of inducing a cholinotoxic syndrome (without inbreeding the animals in advance), and it has all of the major features of the soman cholinotoxic syndrome. In this approach, rats pretreated with li (3 meq/kg) then treated 24 hours later with pilo (30 mg/kg), consistently develop status epilepticus within 10-45 min after pilo. In order to determine whether agents might mimic procyclidine in being effective even when administered after seizure activity is established, we administered various agents ip to li/pilo-treated

animals after seizures had already commenced. Each agent was tested over a range of doses and an ED_{50} (dose arresting seizures and preventing brain damage in 50% of treated rats) was calculated by linear regression analysis. As the results in Table 4 indicate, several agents are more effective than procyclidine in reversing the li/pilo cholinotoxic syndrome when administered after the rats are already seizing.

Our findings suggest that it would be advisable to test such agents as scopolamine, trihexyphenidyl, benactyzine, benztropine and biperiden (Knoll Pharmaceutical) for their ability to prevent the cholinotoxic syndrome induced by soman when administered immediately after onset of soman seizures. Of the anticholinergic drugs evaluated for efficacy in protecting against the li/pilo SRBD syndrome (Table 4), several have been tested for NMDA antagonist activity (scopolamine, trihexyphenidyl, biperiden and procyclidine); in NMDA antagonist activity, procyclidine is the most powerful of these agents and scopolamine is the least powerful (24), but in anti-cholinotoxic activity, procyclidine is the least potent and scopolamine is the most potent. This would suggest that the anti-cholinotoxic activity of these agents does not stem primarily from their NMDA antagonist properties. The most obvious property that the compounds in Table 4 have in common is that they are all anti-cholinergic agents that bind to muscarinic receptors. Two subtypes of muscarinic receptors have been identified, M-1 and M-2. The agents in Table 4 bind to both receptor classes, but their order of potencies as anti-cholinotoxic agents correlates better with their binding affinities for M-1 than M-2 receptors (25). It seems likely, therefore, that a high binding affinity to M-1 receptors may be an important key to the development of effective anti-cholinotoxic drugs.

Summary and conclusions

The findings described and discussed in this report are consistent with the interpretation that the brain damage associated with both the pilo and soman cholinotoxic syndromes (either with or without li pretreatment) is seizure-mediated brain damage which does not occur if seizure activity does not occur or if it is arrested soon after onset. In addition, our findings are consistent with the interpretation that all SRBD syndromes, whether induced by cholinergic or non-cholinergic means, involve excessive activation of EAA receptors as the primary mechanism by which the brain damage occurs. Therefore, a rational prophylaxis against the soman cholinotoxic syndrome might be to efficiently arrest soman-induced seizure activity and/or to block EAA receptors, thereby preventing brain damage even if all seizure activity is not arrested. The latter approach is exceedingly problematic because methods currently available for blocking EAA receptors are associated with multiple serious side effects, including a paradoxical effect whereby the most potent EAA receptor antagonist known (MK-801) exerts an anti-convulsant action through EAA receptors and a proconvulsant action through cholinergic receptors. However, certain anticholinergic agents which have been relatively ignored in prior soman research appear to be quite effective in preventing cholinergically-induced seizure activity or, more importantly, in arresting it after it has already commenced. It seems likely that the ability of these agents to block M-1 muscarinic receptors is the primary basis for their remarkable efficacy in preventing cholinergically-induced seizures and that protection against brain damage is a fringe benefit that automatically follows from the prevention of seizure activity. While it is interesting that some of these agents are NMDA antagonists as well as M-1 muscarinic antagonists, it seems unlikely that antagonism of NMDA receptors plays a major part in their anti-cholinotoxic properties. The most important feature of the findings described herein is the fact that procyclidine and related agents have the ability to totally arrest cholinergically-induced seizure activity even when they are not administered until after seizure activity is manifested. This suggests that they may be very promising drugs for protecting either civilian or military populations against cholinotoxic agents used in chemical warfare.

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FIGURE LEGENDS

Fig. 1 Pattern of electrographic seizure onset in two animals (A and C) treated with li/pilo and one animal (B) treated with high-dose pilo. For comparison, the pattern of electrographic seizure onset from an animal treated with KA (12 mg/kg sc) is shown in (D). The traces display the first organized electrographic seizure in each animal. HC, hippocampus; CTX, motor cortex; SN, substantia nigra; NA, nucleus accumbens; AMY, amygdala; VP, ventral pallidum; EC, entorhinal cortex. Calibration bars: (A) HC = 1.5 mV, CTX = 0.5 mV, SN = 1.0 mV, NA = 0.5 mV; (B) 0.5 mV for all; (C) VP = 1.0 mV, EC = 0.5 mV, HC = 1.0 mV, SN = 0.25 mV; (D) SN = 1.0 mV, NA = 0.5 mV, HC = 1.5 mV. Time = 1 s for all.

Fig. 2 Electronmicrographs from rats treated with pilo (A and C) or soman (B and D) showing edematous degeneration of neuronal dendrites (A and B) and cell bodies (C and D). Note the synaptic contacts between normal-appearing presynaptic axon terminals and grossly swollen post synaptic dendrites (arrow heads in A and B) or cell bodies (arrow head and inset in C). The degenerating cells show clumping of nuclear chromatin and dissolution of cytoplasmic organelles which signifies a relatively advanced stage of degeneration. These are the same kind of changes that glutamate typically causes (A and B x 24000; C and D x 4000; inset x 32000).

Fig. 3 Glu and QNB binding in li/pilo treated brains as compared to binding in control (untreated) brains 4-24 hrs (a), 1-3 wks (b) and 4-8 wks (c) following li/pilo treatment. The experimental data (li/pilo-treated brains) is expressed as percent of control.

Key:

CC	-	Cingulate Cortex	HIPP CA-1	-	CA-1 Region of Hippocampus
FC	-	Frontal Cortex	HIPP CA-2	-	CA-2 Region of Hippocampus
TC	-	Temporal Cortex	HIPP CA-3	-	CA-3 Region of Hippocampus
RSC	-	Retrosplenial Cortex	DG	-	Dentate Gyrus
PC	-	Parietal Cortex	CP	-	Caudate Putamen
IC	-	Insular Cortex	NA	-	Nucleus Accumbens
PYRC	-	Piriform Cortex	NLOT	-	Nucleus of Lateral Olfactory Tract
OC	-	Occipital Cortex	DLT	-	Dorsolateral Thalamus
PRC	-	Perirhinal Cortex	VPT	-	Ventroposterior Thalamus
BA	-	Basal Amygdala	MA	-	Medial Amygdala
			SN	-	Substantia Nigra

Fig. 4 Electrographic recordings from the neocortex, hippocampus and piriform cortex of a rat prior to 125 μg/kg ip soman (A), immediately after seizure activity began (B) and 5 minutes after administration of 75 mg/kg ip procyclidine (C). In C, electrographic seizure activity has disappeared and there was no evidence for return of such activity over the remaining 4 hour recording period. The brain of this animal was entirely normal when examined 4 hrs after soman treatment.

Fig. 5 Medio-cortical region of the amygdala of the adult rat 4 hours following treatment with pilo (A), soman (B) or soman plus procyclidine (C) (administered after seizure activity had begun). Animals A and B displayed persistent seizure activity for several hours and show a typical SRBD lesion pattern sweeping diffusely across the medial and cortical amygdaloid areas. Animal C stopped seizing within 10 min following procyclidine (75 mg/kg ip) and shows no cytopathological changes (x 100).

Figure 1

Figure 2

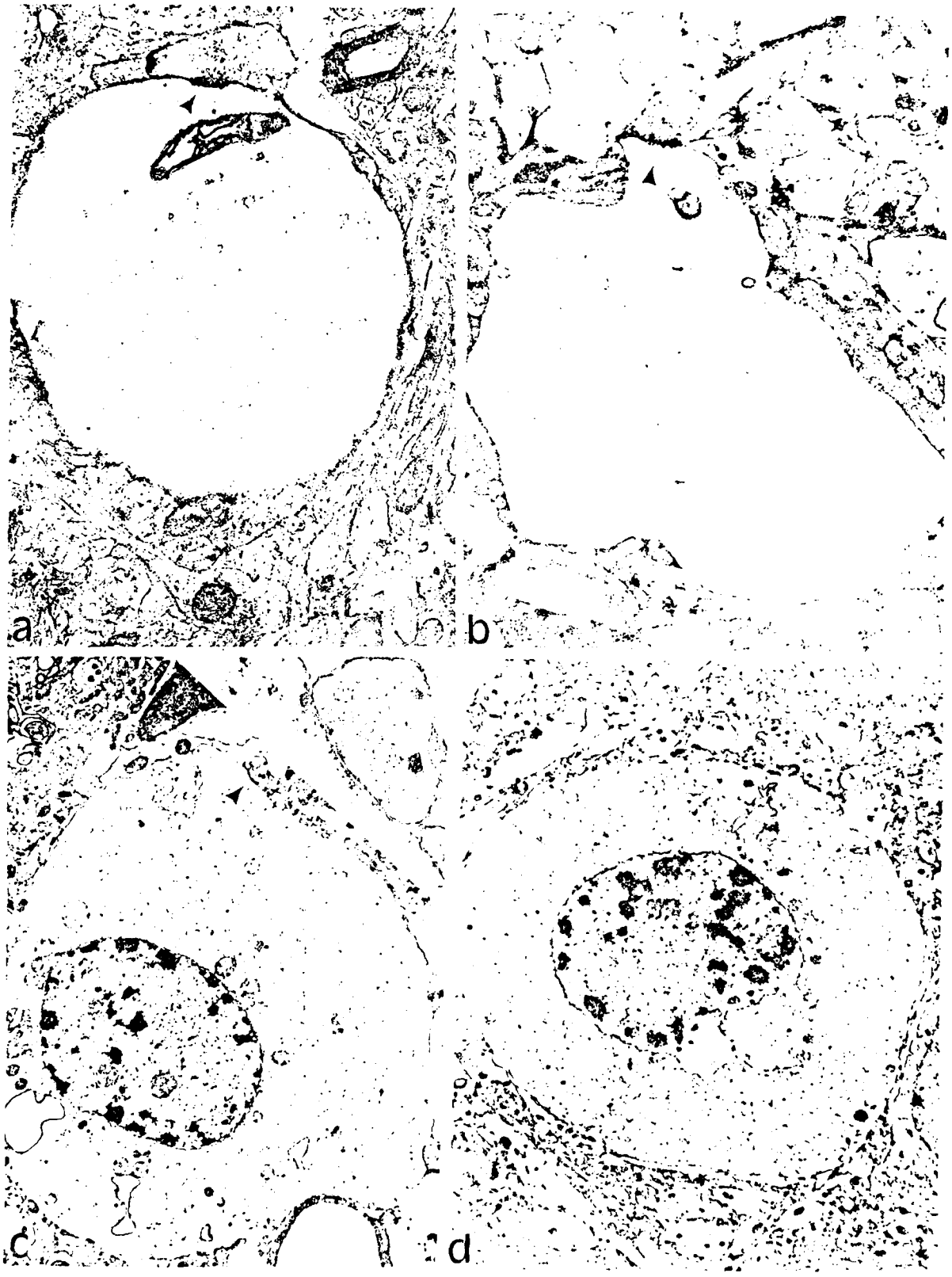
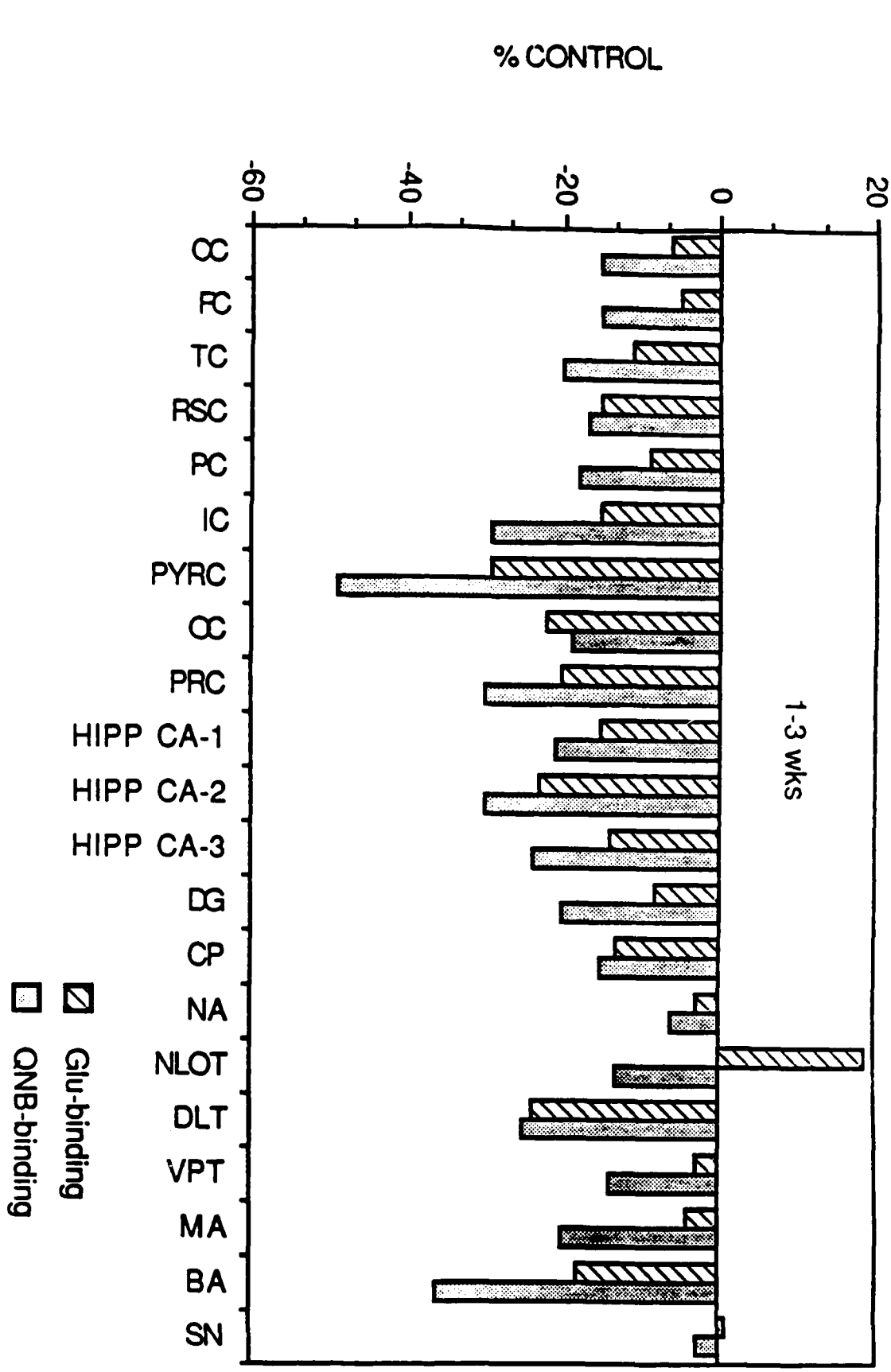


Figure 3A

% CONTROL
10
0
-10
-20
-30
-40
-50
4-24 hrs
CC
RC
TC
RSC
PC
IC
PYRC
OC
PRC
HIPP CA-1
HIPP CA-2
HIPP CA-3
DG
CP
NA
NLOT
DLT
VPT
MA
BA
SN
Glu-binding
QNB-binding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-40
-20
0
20
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FC
TC
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PC
IC
PYRC
OC
PRC
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HIPP CA-2
HIPP CA-3
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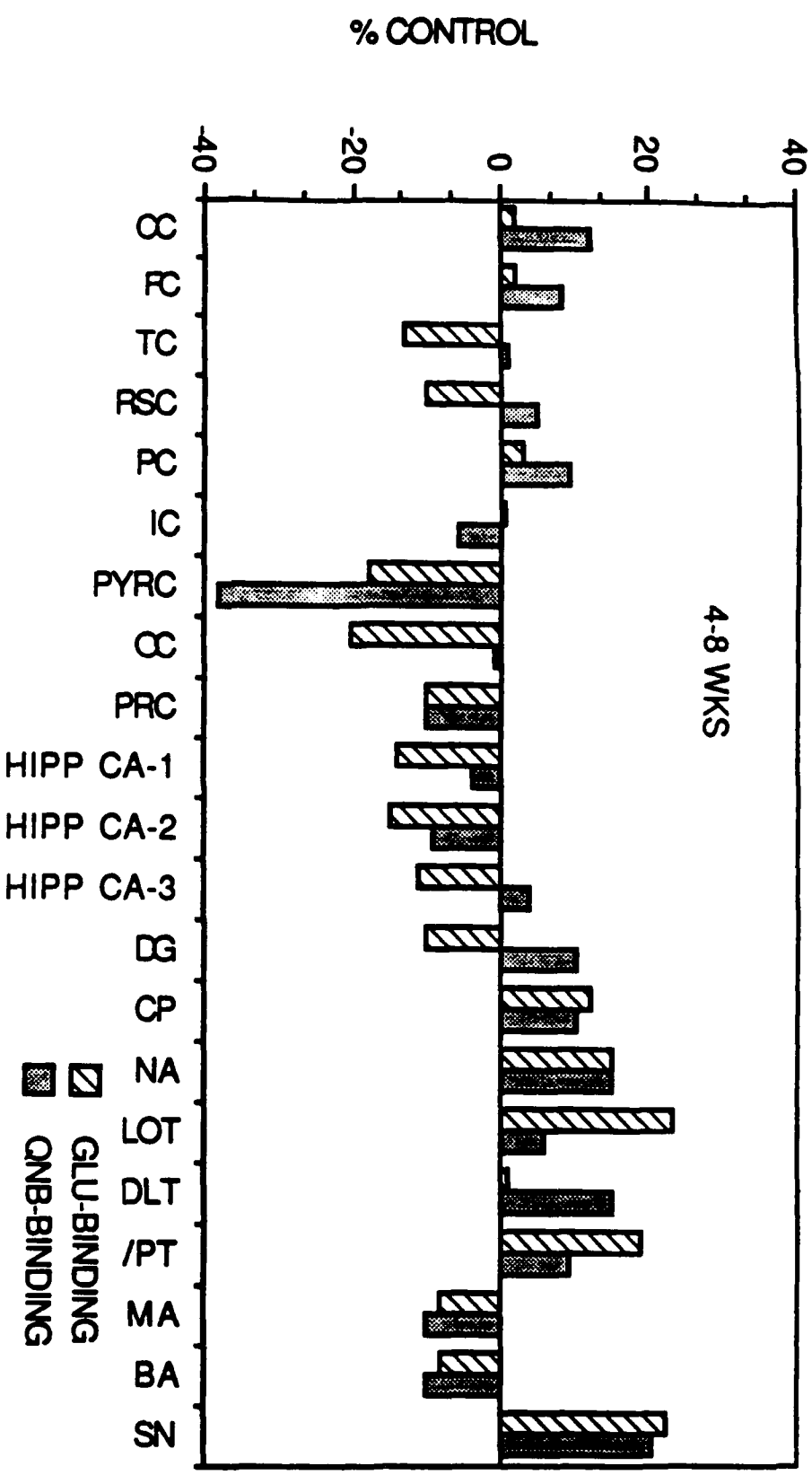
NLOT
DLT
VPT
MA
BA
SN
1-3 wks
QNB-binding
Glu-binding

Figure 3B


% CONTROL
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-20
0
20
40
CC
RC
TC
RSC
PC
IC
PYRC
CC
PRC
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HIPP CA-2
HIPP CA-3
DG
CP
NA
LOT
DLT
/PT
MA
BA
SN
4-8 WKS
QNB-BINDING
GLU-BINDING

Figure 3C

Figure 4

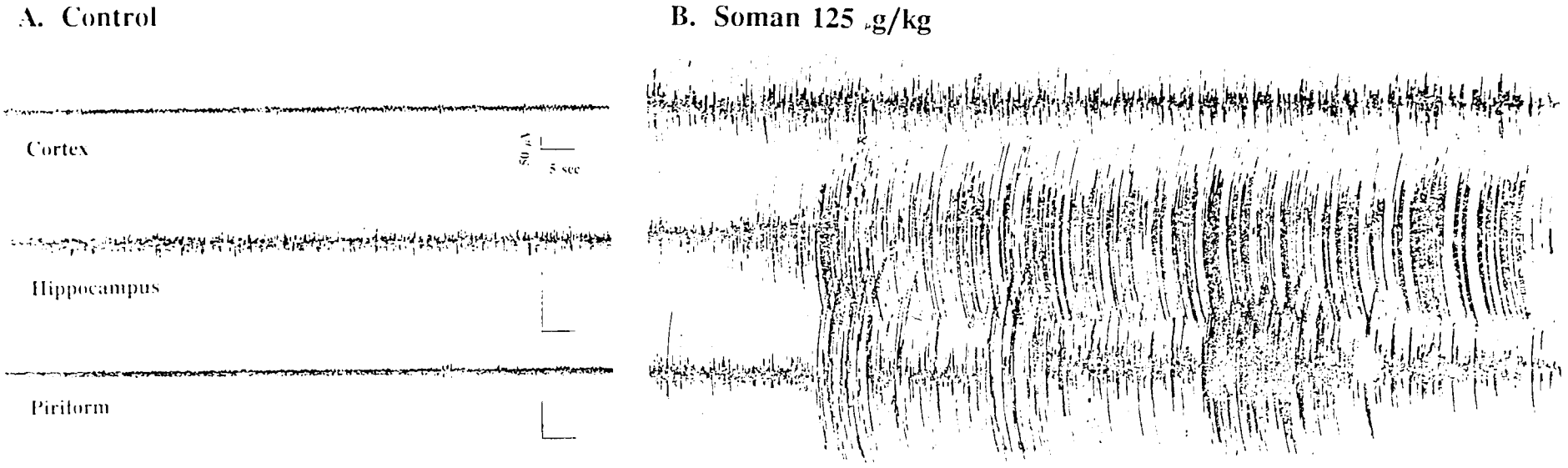
A. Control
Cortex
50 μA
5 sec
Hippocampus
Piriform
B. Soman 125 μg/kg
C. +Procyclidine 75 mg/kg

Figure 5

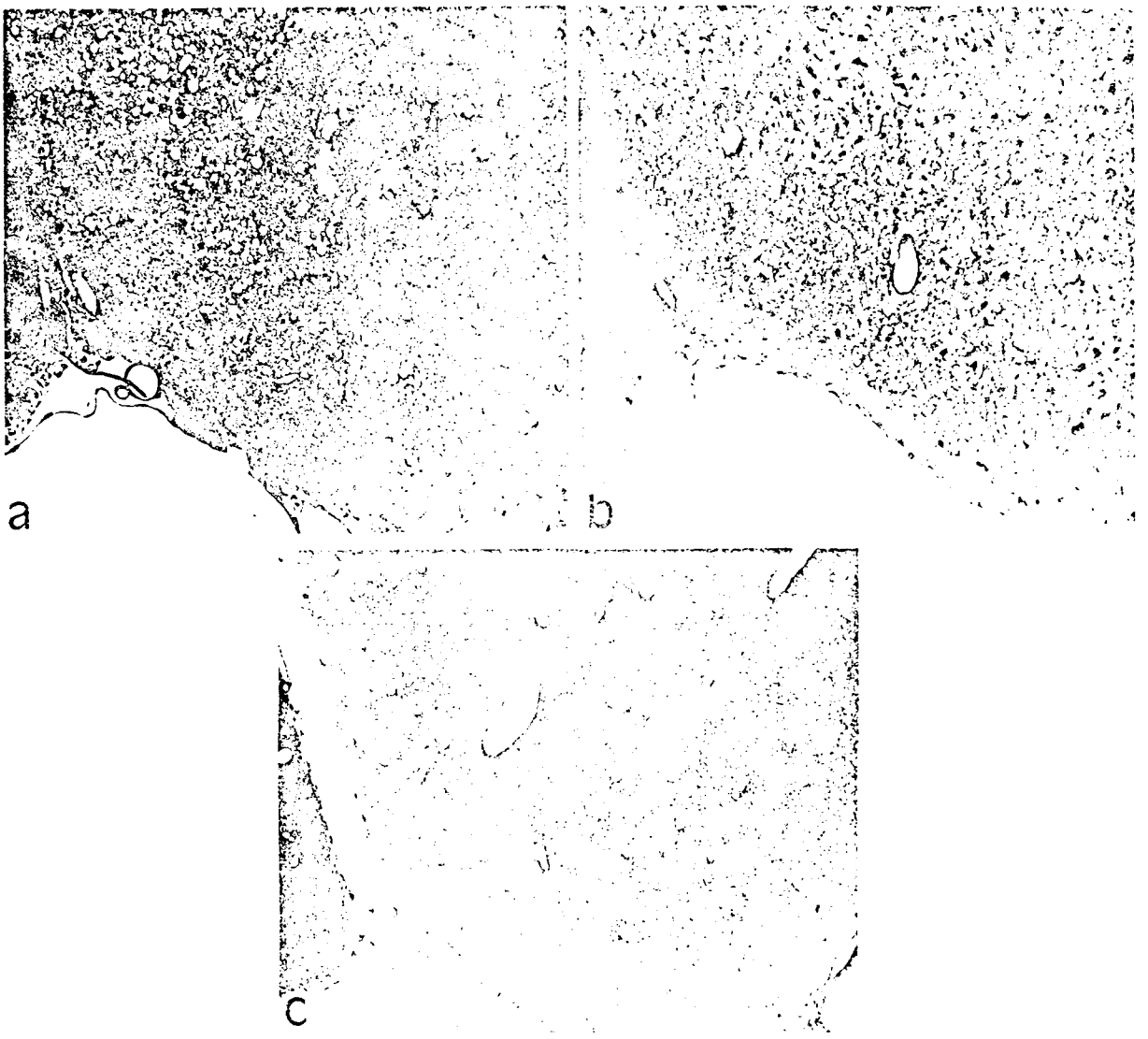

Table 1

Glucose utilization (μmoles/100g/min) in pilo-treated rats expressed as mean ± standard error of the mean (SEM)

	Li/pilo		Control		Pilo	
	Mean	SEM	Mean	SEM	Mean	SEM
Frontal cortex	295.67	25.50	73.77	5.41	323.97	39.57
Caudate	275.77	23.21	83.20	6.30	277.33	40.77
Medial accumbens	174.57	16.75	70.10	7.03	189.70	41.38
Lateral accumbens	175.90	16.23	68.47	5.88	187.80	26.71
Piriform cortex	321.13	21.41	73.00	6.66	312.73	28.10
Corpus collosum	113.13	15.59	17.00	2.47	117.93	16.44
Lateral septum	253.50	20.47	51.03	5.29	275.50	36.67
Medial septum	156.00	14.70	68.60	5.98	173.53	29.61
Ventral diagonal band	138.97	18.49	66.40	3.43	163.57	31.13
Ventral pallidum	289.00	24.19	55.73	7.56	271.42	36.83
Olfactory tubercle	314.17	27.06	73.87	8.10	300.55	42.53
Globus pallidus	236.83	24.35	42.40	4.65	238.20	35.65
Horizontal diagnonal band	144.00	27.25	65.10	4.10	156.30	32.67
Anterior cingulate cortex	240.63	30.30	89.60	2.77	295.17	55.13
Dorsal hippocampus CA1	172.83	13.41	35.03	8.24	249.70	37.43
Dorsal hippocampus perforant path	221.30	18.75	43.87	6.57	263.40	34.52
Dorsal hippocampus dor. dentate	213.27	18.49	47.83	8.71	278.43	37.98
Dorsal hippocampus ventral dentate	208.93	19.83	33.83	3.02	248.03	37.67
Dorsal hippocampus CA3	232.07	17.22	45.47	8.79	250.71	52.30
Amygdala	255.30	20.32	50.10	7.20	284.17	28.90
Ventrobasal thalamus	264.83	17.30	65.97	5.90	333.87	58.17
Medial dorsal thalamus	291.60	23.33	54.13	9.41	326.37	32.48
Sensory cortex	258.60	27.46	93.00	13.47	370.13	55.68
Hypothalamus	135.37	15.61	46.93	6.85	124.50	22.44
Hilum dentate	193.50	22.22	39.33	6.68	229.37	32.07
Habenula	144.00	11.23	62.13	9.56	190.87	27.04
Retrosplenial cortex	203.87	25.64	72.13	7.31	284.80	46.59
Visual cortex	215.93	18.36	68.10	5.80	285.67	44.09
Auditory cortex	235.20	16.71	116.65	5.36	308.33	47.26
Entorhinal cortex	260.90	25.87	43.90	5.96	275.27	22.99
Subiculum	256.97	22.04	46.13	6.88	290.80	29.35
Ventral hippocampus CA1	230.532	14.56	34.30	4.49	263.80	29.86
Ventral hippocampus CA3	242.10	22.47	43.37	6.88	252.37	31.41
Ventral hippocampus dentate	274.43	33.40	52.10	7.11	281.00	39.51
Substantia nigra	271.23	11.83	48.60	4.97	301.57	37.91
Medial geniculate	231.43	23.15	99.47	5.63	317.57	47.13
Dorsal lat. geniculate	69.27	7.72	51.13	6.04	100.57	19.25
Medial posterior thalamus	65.10	2.07	45.03	6.03	77.33	18.47
Superior colliculus	72.53	1.33	65.67	3.80	94.17	23.69
Periaquaductal gray	91.77	4.00	56.47	5.92	119.40	28.36

Table 2

	Glu binding		QNB binding	
	control	Li/pilo	control	Li/pilo
K_d	148±22	242±29*	0.34±0.04	0.31±0.1
B_{max}	20.8±1.25	19.3±0.84	5.25±0.65	3.34±0.39*

K_d (nM) and B_{max} (pmoles/mg prot) of Glu and QNB binding in 6 rats 1-3 wks after li/pilo treatment as compared to age-matched controls. Data are expressed as mean ± SEM. Significant differences ($p<0.05$) between control and experimental data are indicated by asterisks.

Table 3

Concentrations of GAD and CAT (pmoles/mg/min) in adult rats 1-12 wks after li/pilo treatment as compared to age-matched controls. Data are expressed as mean ± SEM. Asterisks indicate significant differences between control and li/pilo data as determined by Student's t tests (95% or greater confidence levels). (N = 6 per group.)

GAD

	1 wk		2 wk		3 wk		4 wk		12 wk	
	control	li/pilo	control	li/pilo	control	li/pilo	control	li/pilo	control	li/ pilo
Striatum	26.1±1.2	26.1±1.3	26.9±0.5	27.9±1.0	25.3±0.7	25.8±1.3	28.9±2.0	24.5±0.9	22.2±1.1	24.2±1.4
Neocortex	25.9±1.8	25.5±1.2	27.1±0.9	28.9±1.3	27.8±1.1	26.9±1.0	29.0±1.5	29.9±1.6	23.0±1.7	27.0±1.8
Hippocampus	26.4±0.9	22.0±1.3*	28.3±0.7	23.4±0.8*	29.3±1.0	24.5±0.8*	26.9±1.0	26.9±1.0	21.7±1.3	23.0±1.1
Piriform cortex and amygdala	29.1±1.3	23.1±4.3*	27.6±0.7	24.7±0.9*	29.2±1.1	22.9±1.3*	27.3±1.3	25.0±1.7	22.8±2.1	26.0±2.5
Thalamus	31.5±1.4	26.9±0.9*	34.2±1.1	28±0.9*	34.2±3.2	31.2±2.0	34.7±6.2	39±3.2	29.5±1.7	27.3±1.3

CAT

Striatum	499±59	384±36*	364±16	278±35*	431±22	355±20*	432±22	306±43*	366±32	305±27*
Neocortex	140±8	206±18*	118±10	140±12	120±19	189±15*	119±22	142±34	103±7	92±8
Hippocampus	183±20	193±15	158±18	134±20	169±15	167±5	161±8	150±14	145±11	138±14
Piriform cortex and amygdala	291±24	220±19*	251±22	172±11*	282±16	223±13*	222±14	175±8*	203±14	173±14*
Thalamus	182±20	184±26	156±14	145±20	195±20	212±27	190±14	162±13	151±12	144±12

Table 4

Drug	ED_{50} (mg/kg)
scopolamine	0.93
trihexyphenidyl	2.5
benactyzine	4.2
biperiden	4.5
benztropine	4.5
procyclidine	8.3
atropine	22.9

Anticholinergic agents listed in order of their ability to block li/pilo-induced SRBD. All drugs were tested over a range of doses and were administered ip immediately after seizure activity had begun. ED_{50} indicates the dose that arrested seizures and prevented brain damage in 50% of animals in a given dose group.

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